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14. ABSTRACT In the C3(1)/SV40 T-antigen (Tag) FVB/N mouse model of human estrogen and progesterone receptor-negative breast cancer, the stress response elicited by social isolation is associated with increased expression of metabolic genes in the mammary gland. To further understand accelerated tumor growth associated with social isolation, we separated mammary gland adipocytes from ductal epithelium and stroma and then analyzed individual fractions for changes in metabolic gene expression and function. The increased expression of the key metabolic genes <i>Acaca</i> , <i>Hk2</i> and <i>Acly</i> was found to be significantly elevated in the adipocytes of the mammary gland, and surprisingly, was not significantly increased in visceral adipose depots of socially isolated female mice. Increased metabolic gene expression in the mammary gland of socially isolated mice coincided with increased glucose metabolism, lipid synthesis, and leptin expression. Furthermore, culture media from isolated versus group-housed mouse mammary adipose tissue resulted in relatively increased proliferation of mammary cancer cells. These results suggest that exposure to chronic social isolation results in metabolic changes in mammary gland adipocytes that contribute to increased growth of adjacent epithelial cell tumors. We propose a model in which environmental stress affects estrogen-independent mammary tumor growth, at least in part, through changes in mammary adipocyte biology.					
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## **Introduction**

In SV40 T-antigen (TAg) FVB/N mice, the chronic stress of social isolation is associated with larger mammary gland tumor burden and increased tumor invasiveness independently of circulating estrogen and progesterone levels and tumor hormone receptor status . We previously reported that chronic stress is also associated with upregulation of lipid synthesis genes and a concomitant increase in de novo lipid synthesis in the mammary adipocytes (and not other adipose depots) of TAg mice . Furthermore, mammary fat culture media from stressed vs. unstressed animals significantly augmented proliferation of TAg-derived cancer cells. Surprisingly, lipid profiling of mammary fat revealed a dramatic increase in lysophosphatidylcholine (LPC) in stressed vs. unstressed mice. Acting as an important lipid precursor, LPC is processed by Autotaxin (ATX), a secreted phospholipase, to generate lysophosphatidic acid (LPA), a signaling lipid that has been implicated in diverse pathological processes, including breast cancer. Hypothesizing that LPC/ATX/LPA-axis activity originating from the local mammary adipocytes influences TAg breast cancer progression, we interrogated the contribution of LPA signaling to the pro-cancer effects of the mammary adipose tissue secretome. Furthermore, we investigated how exposure of mammary fat to TAg epithelium affects the expression of ATX. Our data identify LPA signaling as a significant contributor to the oncogenic effects of the mammary adipose tissue secretome and suggest heterotypic interactions between cancer cells and stromal adipocytes alter the LPA-axis in mammary adipose tissue during breast cancer progression.

## **Body**

Cancer progression involves the interaction between cell-autonomous effects, specified by the genomes of cancer cells, and the paracrine signals derived from the tumor stroma<sup>1</sup>. Over the past decade, increased effort has been placed on identifying stroma-derived signals that contribute to cancer progression. These efforts have sought to identify new targets for cancer intervention and treatment that may be less susceptible to the acquired therapeutic resistance that is common in genetically unstable cancer cells<sup>2</sup>. Importantly, the paracrine interactions that influence a developing cancer are dependent on the unique cellular and structural components of the microenvironment in which the cancer is situated. In breast cancer biology, the mammary microenvironment is increasingly appreciated as unique, in large part due to the embedding of the mammary epithelium within an adipocyte-laden fat depot.

Prior to the adoption of the adipocyte as an endocrine cell, convention branded adipocytes as inert lipid-storage vessels<sup>3</sup>. We now know that adipocytes define a diverse and dynamic cell population with phenotypic properties specific to their location in the body<sup>4</sup>. Adipose tissue depots regulate whole-body metabolism and are capable of dramatically influencing cancer biology<sup>1,5</sup>. Within the mammary microenvironment, local communication between the adipocytes and epithelium regulates normal ductal development<sup>6</sup>, and compelling evidence suggests that mammary adipocyte-derived signals can exacerbate breast cancer progression<sup>7-9</sup>. However, whether environmental factors, such as social stress, can influence the oncogenic contribution of local mammary adipocytes has been largely unexplored.

In SV40 T-antigen (TAg) FVB/N mice, the chronic stress of social isolation is associated with larger mammary gland tumor burden and increased tumor invasiveness independently of circulating estrogen and progesterone levels and tumor hormone receptor status<sup>10</sup>. We previously reported that chronic stress is also associated with upregulation of lipid synthesis genes and a concomitant increase in de novo lipid synthesis in the mammary adipocytes (and not other adipose depots) of TAg mice<sup>11</sup>. Furthermore, mammary fat culture media from stressed vs. unstressed animals significantly augmented proliferation of TAg-derived cancer cells<sup>11</sup>. Surprisingly, lipid

profiling of mammary fat revealed a dramatic increase in lysophosphatidylcholine (LPC) in stressed vs. unstressed mice.

LPC is a phospholipid precursor to the important signaling molecule, lysophosphatidic acid (LPA)<sup>12</sup>. LPA acts through specific G-protein-coupled receptors and has been implicated in diverse pathological processes, including breast cancer<sup>12</sup>. Conversion of LPC to LPA is mediated by Autotaxin (ATX), a secreted phospholipase. Increase LPA signaling is believed to contribute to cancer cell proliferation, survival, invasion, and metastasis<sup>12</sup>.

Breast cancer patients with high ATX expression in their tumors tend to have more aggressive tumors<sup>13</sup>. Indeed, pre-clinical studies targeting ATX expression in cancer cells have reported significant anti-tumor effects<sup>14-16</sup>. However, at least one study has reported significant therapeutic benefit from targeting ATX in a breast cancer model that does not express appreciable ATX<sup>17</sup>. Interestingly, these investigators noted substantial ATX expression in the tumor stroma, although whether antagonism of stroma-derived ATX contributed to their results was not experimentally tested. Furthermore, although breast cancer cells can produce ATX, adipocytes are believed to be the major source of circulating ATX and the major regulator of plasma LPA concentrations<sup>18</sup>. These data suggest that the mammary stroma may significantly contribute LPA-axis signals and/or be perturbed in the presence of a developing cancer. Taken together, these observations support further investigation into the contribution of mammary adipocyte-derived LPA-axis signaling to the oncogenic milieu of the adipocyte secretome.

To evaluate the hypothesis that LPA-axis activity originating from the local mammary adipocytes can influence TAg cancer progression, we interrogated the contribution of LPA signaling to the pro-cancer effects of the mammary adipose tissue secretome. We also investigated how exposure of mammary fat to cancerous epithelium affects LPA-axis regulation in the mammary adipose tissue. Our data identify LPA signaling as a significant contributor to the oncogenic effects of the mammary adipose tissue secretome and suggest heterotypic interactions between TAg cancer cells and stromal adipocytes alter the LPA-axis in mammary adipose tissue during breast cancer progression.

*LPC and LPA are detected in mammary adipose CM and TAg cells lines express LPA receptors-* To investigate the role of mammary adipocyte derived LPA signaling on breast cancer biology, we chose a progressive series of breast cancer cell lines (M28N2, M27H4, and M6) derived from the SV40 T-antigen (TAg) model of triple negative breast cancer<sup>19</sup>. M28N2, M27HR, and M6 cells (referred to in this text as normal, CIS, and invasive, respectively) were isolated from morphologically normal epithelium, carcinoma in situ, and an invasive tumor, respectively (Fig. 1A)<sup>19</sup>. These cells are unresponsive to estrogen and display properties, both in vitro and as xenografts, which are consistent with their representative stage of tumor progression<sup>19</sup>. The TAg cell series models the progression of TAg-cancers *in vivo*.

Conditioned media experiments have revealed that adipocyte secreted factors can support the proliferation of breast cancer cells and contribute to their aggressiveness<sup>20-22</sup>. This approach has helped to identify adipokines, such as Leptin and hepatocyte growth factor, as putative factors that contribute to breast cancer progression<sup>23,24</sup>. However, adipocytes secrete a plethora of growth factors, cytokines, and metabolites, and their individual effects likely converge to produce the pro-tumorigenic effects of adipose tissue conditioned media on cancer cells<sup>25</sup>. Furthermore, adipose depots display metabolic and secretory properties that are specific to their physiological location<sup>26</sup>. Therefore, we next sought to develop a conditioned media system that would best represent the mammary microenvironment and would allow us to determine whether LPA signaling is an important contributor to the overall pro-tumorigenic effects of adipose tissue secreted factors.

To model the oncogenic effects derived from adipocytes of the mammary microenvironment, we harvested primary mammary fat pads from TAg mice, enriched for mammary adipose tissue, and made conditioned media. We used a relatively short (8hr) culture period for conditioned media preparation to help minimize the effects of autocrine signaling and to preserve labile unsaturated lipids secreted into culture media. This conditioned media was sufficient to support the proliferation and survival of the TAg breast cancer cell lines (Fig. 1B). We measured LPC and LPA concentrations in the 8hr

conditioned media (Fig. 1C). After 8hrs, LPC concentration in the CM were roughly 1uM and LPA concentrations averaged about 180nM.

LPA has been shown to interact with at least 6 known G protein-coupled receptors (LPA<sub>1-6</sub>). We began by evaluating in the TAg cell lines the mRNA expression of the “classical” LPA receptors (LPA<sub>1-3</sub>), which have been implicated in breast cancer progression<sup>27</sup>. Transcripts for all three receptors were detected to varying degrees in all cell lines; however, LPA<sub>2</sub> mRNA was the most abundant within each individual cell line (Fig. 1D). We were unable to determine protein level expression of the LPA<sub>Rs</sub> due to ineffective antibodies. These data indicate that TAg breast cancer cells express receptors for LPA and that LPA and its precursor are abundant in mammary adipose conditioned media. These observations are in line with the hypothesis that LPA signaling contributes to the oncogenic effects of the mammary adipose secretome.

*LPA is a proliferative and survival signal for TAg cancer cells-* LPA has been reported to stimulate proliferation and survival of numerous breast cancer cell lines<sup>28</sup>, but whether or not TAg cells are responsive in a similar manner had not been reported. To determine whether LPA signaling affects cell proliferation and/or death in TAg cells, we cultured the cell lines in the absence of serum and monitored their proliferation and cell death in response to LPA dosing. We chose LPA doses that encompassed the concentrations of both LPA (nM) and LPC (low uM) in the CM. This was because the abundance of adipocytes-derived ATX in the mammary CM and/or action of TAg cell-derived ATX allows for LPC to exist as a readily available pool of LPA precursor. In the absence of serum factors for a 48hr period, all cell lines underwent appreciable cell death and were unable to sustain proliferation; however, the addition of LPA dose-dependently stimulated TAg cell proliferation and protected from cell death (Fig. 2A,B). To determine the responsiveness of TAg cells to LPA, we next stimulated the cells with increasing doses of LPA and monitored phosphorylation of AKT, a downstream target of LPA signaling (<sup>29</sup>). All cell lines displayed dose-responsive AKT phosphorylation starting in the low nM range and maxing out around 2.5uM (Fig. 2C). These data indicate that



LPA, at concentrations well within range of levels measured in mammary CM, activates proliferative and survival signaling in TAg cell lines.

*LPA signaling through LPA receptors LPAR1/3 significantly contributes to the oncogenic effect of the mammary adipose tissue secretome.* If LPA signaling is a significant contributor to the pro-tumorigenic effects of the mammary adipose tissue secretome, we hypothesized that antagonizing LPA signaling in TAg cells cultured with mammary adipose conditioned media would attenuate the secretome's proliferative and survival effects. We used two small molecules to antagonize LPA signaling; H2L5186303, which is specific to LPAR2<sup>30</sup>; and Ki16425, which targets both LPAR1 and LPAR3<sup>31</sup>. With the exception of the 10uM dose, which may have led to non-specific cell death in the invasive cells (data not shown), antagonism of LPAR2 did not attenuate the proliferation of any of the TAg cell lines (Fig. 3A). Conversely, the two least aggressive cell lines were acutely sensitive to increasing doses of the antagonist targeting LPAR1/3 (Fig. 3B). The effects on the conditioned media in these cells were almost completely ablated at the 10uM concentration; however, cell death data suggest that off-target effects may occur at this high concentration of antagonist (data not shown). Interestingly, although the invasive cell line was responsive to exogenous addition of LPA this cell line was not affected by either of the small molecules (Fig. 3A,B).

To determine the importance of individual receptors in mediating the pro-tumorigenic effects of conditioned media, we next performed siRNA-mediated knockdown of each receptor in the cell lines that responded to LPAR1/3 antagonism (normal and CIS). Knockdown of LPAR1 and LPAR3 was validated by quantitative real-time PCR; however, we were unable to successfully knockdown LPAR2 expression in either cell line (data not shown). In the normal cell line, knockdown of either LPAR1 or LPAR3 significantly attenuated the proliferative and survival effects of the CM, although the effects of LPAR1 knockdown were more pronounced (Fig. 4A). Conversely, in the CIS cells, knockdown of LPAR3 had no effect, whereas LPAR1 knockdown dramatically antagonized the ability of the CM to promote proliferation and cell survival (Fig. 4B). These data are in line with the results obtained during pharmacologic inhibition of

LPA<sub>r1/3</sub> and suggest that LPA signaling dynamics may change during cancer progression.

*LPC can support TAg cell proliferation independent of LPA signaling.* Although adipocytes are believed to be the major source of ATX in circulation<sup>18</sup>, TAg cancer cells also express ATX; the highest expression in the normal cells, intermediate expression in the CIS cells, and lowest expression in the invasive cells (Fig. 5A). Because the LPA precursor, LPC, is also released by mammary adipocytes, we hypothesized that cancer-cell autonomous conversion of mammary adipocyte-derived LPC to LPA may contribute to the cancer-promoting effects of the adipocyte secretome. To determine whether LPC is sufficient to promote the proliferation and survival of TAg cancer cells, we stimulated TAg cells with increasing doses of LPC (0-40uM) under serum free conditions. The normal and invasive TAg cells were responsive to LPC dosing; however, LPC doses did not augment proliferation in the CIS cells (Fig. 5B). Interestingly, ATX expression in the cell lines did not correlate with LPC responsiveness because the LPC-responsive invasive cell line had the lowest mRNA and protein expression of ATX (Fig. 5A). This suggested that the invasive cell line may benefit from LPC independent of LPA signaling. To test this hypothesis, we stimulated the LPC-responsive cell lines (normal and invasive) with LPC in the presence or absence of PF-8380, a small molecule ATX antagonist<sup>32</sup>. Inhibition of ATX activity eliminated the proliferative effect of LPC in the normal cell line. Conversely, ATX inhibition did not alter the ability for LPC to promote cell proliferation in the invasive cells. These data suggest that mammary adipocyte derived LPC can support subsets of cancer cells with high ATX expression by serving as an LPA precursor, but can also support subsets of highly aggressive cancer cells independent of LPA-axis activity.

*Cancerous epithelial cells induce ATX expression in mammary adipose tissue.* A recent investigation reported high stromal ATX expression in a subset of breast cancer patient samples and a separate study suggested that grafting of breast cancer cells to the

mammary fat pad of mice was associated with ATX induction within the mammary gland stroma<sup>13,17</sup>. To determine whether stromal ATX expression was associated with cancerous epithelium in TAg mice, we performed anti-ATX IHC on mammary fat pads harvested from 15 week old TAg mice. We observed strong positive ATX staining in the epithelium of the ducts and in situ cells (Fig. 6A). Interestingly, mammary fat pads from TAg mice showed strong ATX staining within the stroma including surrounding the mammary adipocytes (Fig. 6A). These data suggest that cancer-derived signals may induce ATX expression in stromal adipocytes. To test this hypothesis, we generated CM from each of the TAg cell lines, applied the CM from each line separately to mammary adipose tissue, and monitored mRNA and protein expression of ATX following 24hrs in culture. Exposure for 24hrs to the soluble factors derived from TAg cancer cells resulted in upregulation of ATX mRNA and increased ATX protein expression in mammary adipose tissue (Fig. 6 B,C). These data suggest that TAg-cancer-derived factors augment ATX expression in mammary adipose tissue.

## **Key Research Accomplishments**

- We have identified that metabolic gene changes associated with social isolation and increased tumor burden in female mice occur within the mammary adipocytes.
- We have determined that the metabolic gene expression changes in mammary adipocytes and associated with social isolation are not dependent on the background mouse strain.
- We have observed that gene expression changes are specific to the adipocytes of the mammary gland and are not observed in other fat depots.
- The gene expression changes observed in mammary adipocytes of socially isolated animals correlate with functional metabolic changes including increased glucose consumption and increased lipid synthesis.
- In addition to metabolic changes in mammary adipocytes of socially isolated animals, we have observed elevated levels and secretion of Leptin protein in isolated vs. grouped animals' mammary adipose tissue.
- Mammary fat conditioned media from social isolates potentiates the proliferation of cancer cells compared to media made using grouped animal mammary fat, suggesting adipocyte secreted proteins/metabolites are linked to the increased tumor burden observed in vivo.
- The findings detailed above have been compiled into a manuscript that was accepted for publication in Cancer Prevention Research.
- We have further characterized the metabolic gene expression changes within the mammary adipose tissue of social isolates and have identified upregulation of

CHREBP $\alpha/\beta$  as potentially import transcription factors coordinating increased lipid synthesis of specific lipid species within the mammary fat of social isolates.

- We have performed lipidomics profiling of lipids extracted from the mammary adipose tissue of isolated vs. grouped mice and have identified dramatic changes in several polar and neutral lipid species within social isolates.
- Lysophosphatidylcholine (LPC), the major precursor to the important signaling lipid, lysophosphatidic-acid (LPA), is significantly increased in social isolates' mammary fat pads.
- Mammary fat conditioned media has phospholipase-D activity, thus can convert LPC to LPA.
- LPA protects SV40-TAg breast cancer cells from serum starvation-induced apoptosis and allows for proliferation in the absence of serum factors.
- LPA in mammary fat conditioned media significantly contributes to conditioned media-induced TAg cell proliferation
- TAg cancer cells can respond to LPC by ATX-mediated conversion to LPA or (in aggressive TAg cells) through mechanism independent of LPA signaling.
- Exposure of the mammary fat pad to TAg cancer cell-secreted factors induces ATX in the mammary fat pad

## **Reportable Outcomes**

### **Presentations:**

Chronic social stress in a mouse model of triple-negative breast cancer is associated with oncogenic changes in mammary fat. Volden PA, Wonder E, Skor M, Patel F, Conzen SD, Brady MJ. AACR 2013, April 6-10 in Washington, DC.

Mammary adipocyte-specific alteration are associated with paracrine effects on mammary tumorigenesis. Volden PA, Wonder E, Brady MJ, McClintock MK, Conzen SD. AACR 2012, April 2-6 in Chicago, IL

Determining Fatty Acid Aliphatic Chain length by Isotropic Mixing Sachleben JR, Yi R, Volden PA, Conzen SD. Experimental NMR, April 2012 in Miami, FL Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

Mammary adipocyte-specific metabolic alterations are associated with paracrine effects on mammary tumorigenesis. Paul A. Volden, Erin L. Wonder, Maxwell N. Skor, Christopher M. Carmean, Honggang Ye, Masha Kocherginsky, Eleanor Smith, Steven Kregel, Martha K. McClintock, Matthew J. Brady, and Suzanne D. Conzen. Keystone: Cancer metabolism, 2010, Banff, British Columbia

Environmental stress and breast cancer biology: What is the link? Conzen SD, Volden PA, Brady MJ, McClintock MK. AACR 2011: 102nd Annual Meeting, April 2-6 in Orlando, FL

## Publications:

Determining Fatty Acid Composition of Metabolomic Samples by  $^1\text{H}$  NMR Spectroscopy. Sachleben JR, Yi R, **Volden PA**, Conzen SD. JNMR (Under review).

Chronic social isolation is associated with metabolic gene expression changes specific to mammary adipose tissue. Volden PA, Wonder EL, Skor MN, Carmean CM, Ye H, Kocherginsky M, Kregel S, McClintock MK, Brady MJ, Conzen SD. Cancer Prevention Research. In press

The Influence of Glucocorticoid Signaling on Tumor Progression. Volden PA, Conzen SD. Invited mini-review. Brain, Behavior, and Immunity. Brain Behav. Immun. 2013;30 Suppl:S26-31.

## Employment:

Paul Volden recently received an offer for a postdoctoral research position in the lab of Dr. Phillip Scherer at UT Southwestern.

## **Conclusions**

Following the initial observation that the chronic stress of social isolation is associated with increased tumor burden in TAg mice<sup>10</sup>, we have determined that the increased expression of lipid synthesis genes that precedes larger tumor formation occurs within the mammary adipocytes and not the precancerous epithelium<sup>11</sup>. The observation that mammary fat secreted factors from stressed vs. unstressed mice potentiates the proliferation of TAg cancer cells suggests that soluble factors secreted from the mammary adipocytes of stressed mice contribute to larger tumor formation. Although the secreted factor(s) from the mammary fat of stress mice that contribute to increased TAg cancer cell proliferation are still under investigation, we have identified mammary adipocyte-derived LPA-axis activity as one potential mechanism through which the mammary adipocytes of stressed animal contribute to larger tumor formation. Within the mammary fat pad of social isolates, we observed a dramatic increase in LPC. Because LPC is the major precursor to LPA and adipocytes are the predominant source of ATX, we explored the importance of LPA signaling to the cancer-promoting effects of the mammary adipose tissue secretome.

Our data identify LPA signaling as a significant contributor to the ability of mammary adipose tissue CM to induce cancer cell proliferation and inhibit apoptosis. Furthermore, our data suggest secreted factors from cancerous epithelium upregulate the expression of stromal ATX, effectively increasing the potential for mammary adipose tissue to supply LPA to a developing tumor. Taken together, this research emphasizes the mammary adipocytes as fat cells of a distinct adipose tissue depot. Perturbation of mammary adipocytes (separate from or associated with obesity) can influence breast cancer biology. Additionally, because of their proximity, mammary adipocytes uniquely interact with the mammary epithelium to contribute to cancer progression.

Although the precise experimental approaches are still being considered, future studies will seek to investigate the contribution of LPA signaling to increased tumor burden in TAg social isolates. One approach could involve antagonizing LPA signaling through receptors with a small molecule targeting specific receptors. However, this approach would be cancer-centric, therefore likely susceptible to cancer cells acquire



resistance to the specific treatment <sup>33</sup>. An alternative approach that may prove more effective would be to target ATX instead of the LPA receptors. This approach would target cancer cells that increase LPA signaling by upregulating ATX expression, but also potentially antagonize ATX contributed by the stromal adipose tissue, which upregulates ATX expression in the presence of cancer cell secreted factors. An ATX inhibitor would also be expected to diminish systemic ATX/LPA contributions to tumorigenesis <sup>18</sup>. Notably, for any approach that targets ATX in TAg mice, because you will expect dramatic systemic and local changes to components of the LPA-axis, it will be a challenging task to tease out where (cancer cells, stroma, and/or circulation) important ATX antagonism is taking place. Regardless, the potential to target the cancer cells and pathologic interactions with their microenvironment support further investigation into ATX inhibition as a breast cancer therapeutic.

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## Appendices:

### Figure Legends

Figure 1. The mammary adipose tissue secretome supports the proliferation of TAg breast cancer cells and contains abundant LPC and LPA. **A.** Diagram of progressive series of TAg cell lines. **B.** Proliferation of TAg breast cancer cells in response to mammary adipose tissue CM. **C.** Concentrations of LPC and LPA in 8hr CM. **D.** Relative mRNA expression of classical (*Edg*) LPA receptors in TAg cancer cells.

Figure 2. LPA signaling dose-dependently stimulates the proliferation and prevents cell death of TAg cells deprived of growth factors. TAg cells were cultured in the absence of serum and were dosed with the indicated concentrations of LPA. Following a 10 minute incubation, either protein was harvested from the cells for western analysis or the cells were placed in the Incucyte™ live content imaging system for the duration of the experiment. **A.** Proliferation of TAg breast cancer cells in response to dosing of LPA. **B.** Cell death measurement in TAg cells in response to LPA dosing. To control for the influence of total cell number on the count of dead cells, the dead counts (YOYO-1 positive) were normalized to the respective well confluence. **C.** LPA dose response monitoring the phosphorylation of AKT, a downstream target of LPA signaling.

Figure 3. LPA signaling significantly contributes to cancer-promoting effects of mammary adipose CM. Following seeding and overnight culture in DMEM high glucose media with 10%FBS, TAg breast cancer cells were washed and the media was changed to 8hr mammary adipose tissue CM with <0.01% YOYO-1 iodide in DMSO. Cells were then given their respective treatments and doses and then were cultured in the Incucyte™ live content imaging system for the duration of the experiment. **A.** Proliferation of TAg cells in mammary adipose tissue CM during LPAR2 antagonism with H2L5186303. **B.** The effect of Ki6425, Proliferation of TAg cells in mammary adipose tissue CM during LPAR1/3 antagonism with Ki6425.

Figure 4. Knockdown of LPAR1/3 in normal and CIS cells attenuates the proliferative effect of mammary adipose tissue CM. TAg breast cancer cells were reverse transfected with siRNA pools targeting LPAR1 or LPAR3. Following 48hrs of culture, RNA was harvested from a subset of cells to validate mRNA knockdown of target transcripts. For the remaining cells, the media was changed to mammary adipose tissue CM with <0.01% YOYO-1 iodide in DMSO. Cells were then cultured in the Incucyte™ live content imaging system for the duration of the experiment. **A.** Proliferation and cell death of normal TAg cells cultured in mammary adipose CM during knockdown of LPAR1 or LPAR3. **B.** Proliferative effect of mammary adipose tissue CM in TAg cells. **C.** Proliferation and cell during knockdown of LPAR1 or LPAR3 in CIS TAg cells cultured in mammary adipose CM.

Figure 5. LPC can support the proliferation of TAg breast cancer cells. **A.** Relative ATX (Enpp2) mRNA expression (upper) and protein expression (lower) in TAg cell lines. **B.** Proliferation of TAg breast cancer cells in response to dosing of LPC. **C.** Effect of PF-8380 (ATX small molecule antagonist) on the ability of LPC to support normal TAg cell proliferation. **D.** Effect of PF-8380 on the ability of LPC to support CIS TAg cell proliferation.

Figure 6. Exposure to TAg cancer cell factors induces ATX expression in mammary adipose tissue. **A.** ATX IHC of sections from TAg mice mammary glands at 15wks of age showing positive ATX staining (brown) in the mammary epithelium, CIS, and the stromal adipocytes. CM was generated for each TAg cell line by harvesting media from 100% confluent cells following 24hr culture in 5% FBS containing DMEM high glucose. CM from each cell line was then individually applied to minced mammary adipose tissue explants for 24hrs, followed by RNA and protein harvest. **B.** ATX (Enpp2) mRNA expression in mouse mammary adipose tissue following 24hr culture in TAg CM. **C.** ATX protein expression in mouse mammary adipose tissue following 24hr culture in TAg CM.

Figure 1.

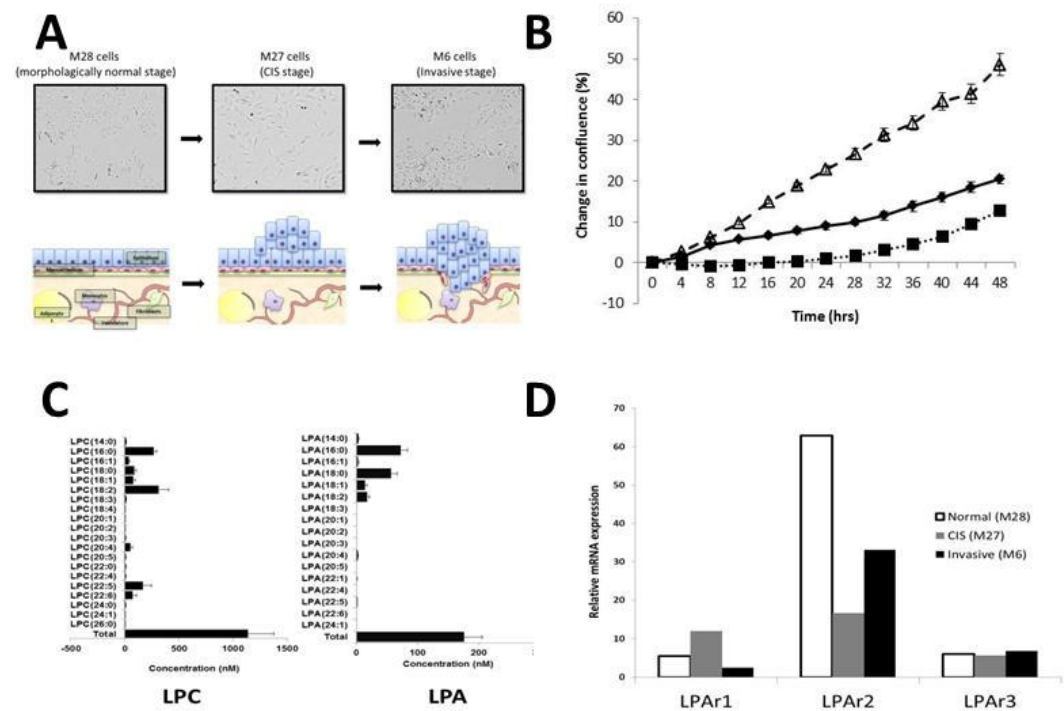


Figure 2.

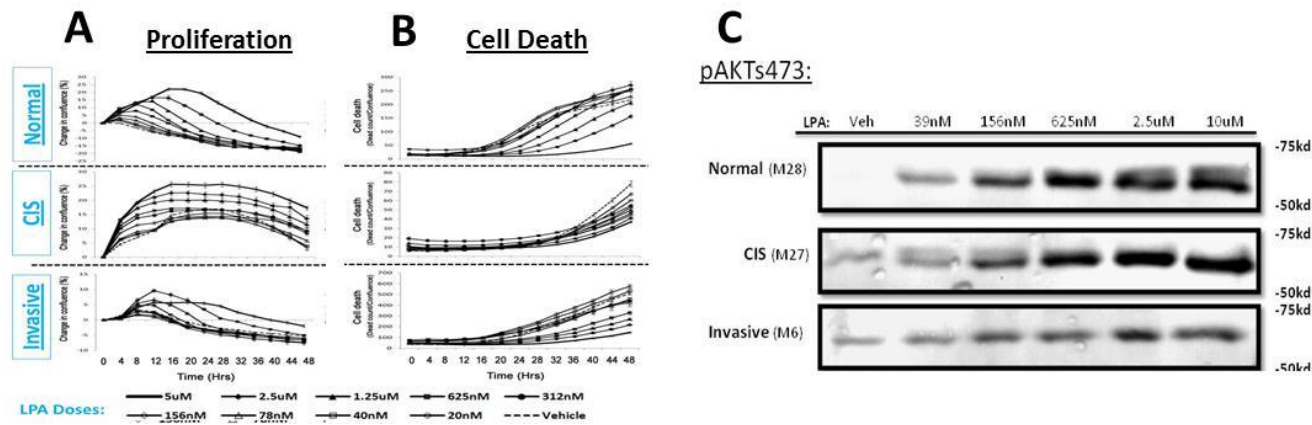


Figure 3.

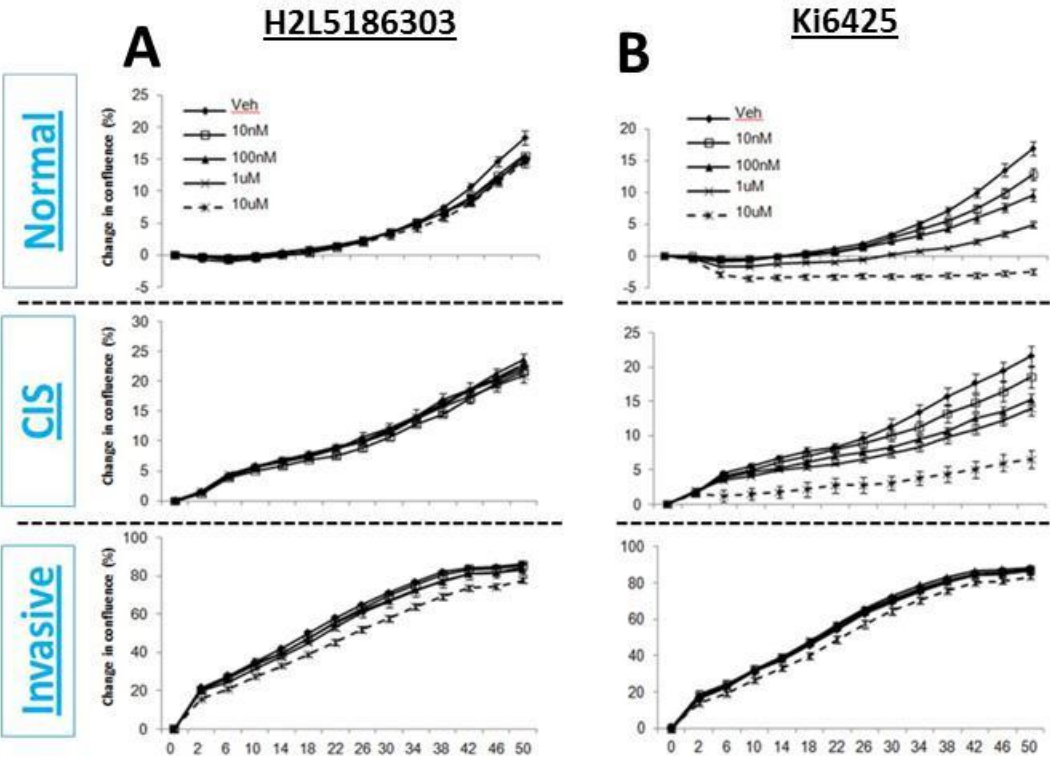


Figure 4.

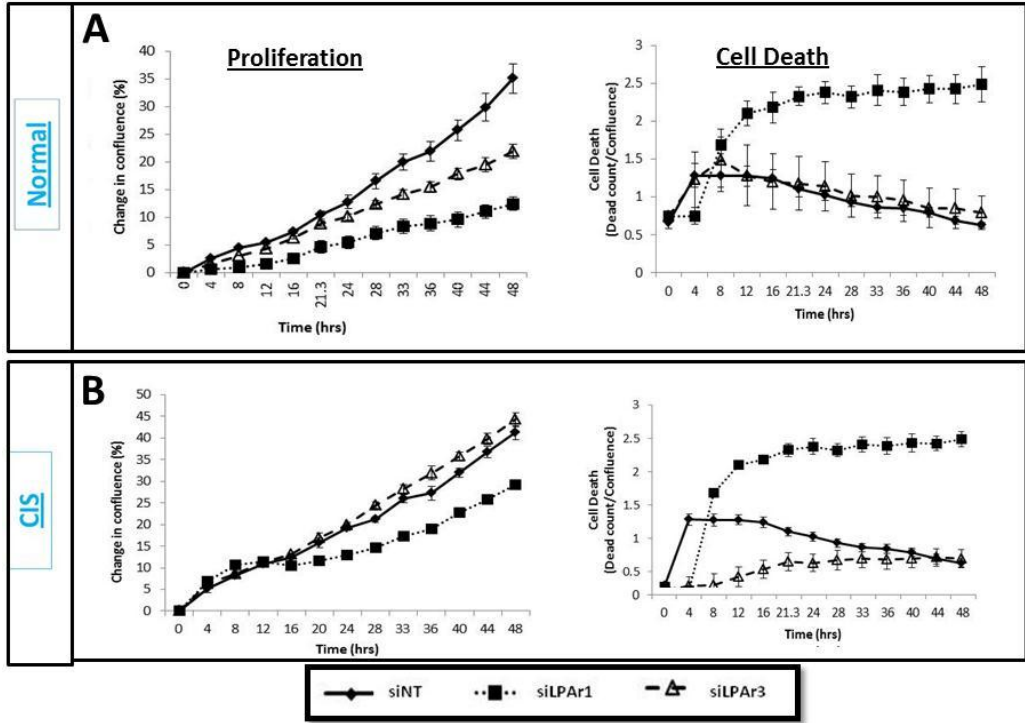


Figure 5.

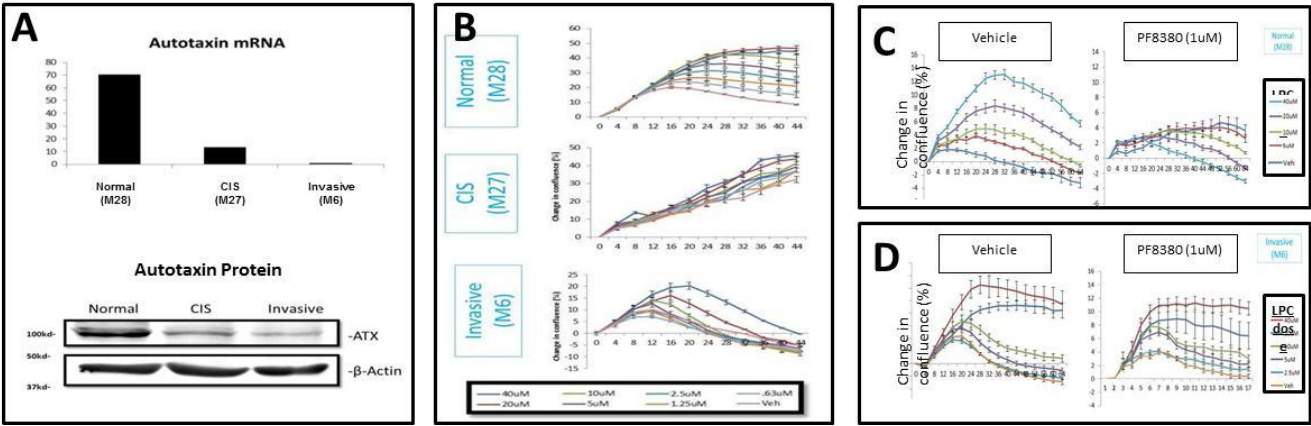


Figure 6.

